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Interaction between inhibitors of inducible nitric oxide synthase and cyclooxygenase in Brewer's yeast induced pyrexia in mice: An isobolographic study

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Abstract

We studied the interaction of *S*-methylisothiourea (a selective inducible nitric oxide synthase inhibitor) with rofecoxib (selective cyclooxygenase-2 inhibitor) and mefenamic acid (non-selective cyclooxygenase inhibitor) in Brewer's yeast-induced pyrexia in mice by isobolographic analysis. Each drug was effective in reducing pyrexia when used alone. Log–dose–response curves of all the three drugs did not show any significant departure from parallelism indicating thereby, a common mode of antipyretic action. However, rofecoxib exhibited significantly higher potency than *S*-methylisothiourea. Isobolographic analysis of combination of *S*-methylisothiourea with rofecoxib and mefenamic acid revealed additive interaction. Experimental ED_{50} of the combinations was not significantly different from theoretical additive ED_{50} of the corresponding drug combination, that substantiated the additive nature of interaction between inducible nitric oxide synthase and cyclooxygenase in Brewer's yeast-induced fever in mice. Results suggest involvement of a mediator that is subservient to both inducible nitric oxide synthase and cyclooxygenase-2 enzyme activities. For further investigation, peroxynitrite ion may be considered to be the putative mediator.

Keywords: Cyclooxygenase; Inducible nitric oxide synthase; Pyrexia; Interaction; Isobologram

1. Introduction

Realizing the significance of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in inflammation, a number of investigations were carried out to unravel the roles of these enzymes in the production of fever. Injection of L^{G}-nitro-L-arginine methyl ester (a non-specific NOS inhibitor) directly into the circulation was reported to attenuate lipopolysaccharide (LPS)-induced fever in rats (Scammel et al., 1996). In another study, aminoguanidine and *S*-methylisothiourea (both predominant inhibitors of iNOS) were shown to produce dose-dependent attenuation of LPS-induced fever in guinea pigs (Roth et al., 1999). Same workers had earlier reported the role of nitric oxide in interleukin-1-beta-induced fever in rats (Roth et al., 1998a). In spite of majority of studies indicating the involvement of nitric oxide in the production of fever, reports suggesting antipyretic roles for nitric oxide do exist in literature. Pharmacological evidence that nitric oxide can act as an endogenous antipyretic factor in endotoxin-induced fever in rabbits was put forth long before (Gourine, 1995). Role of nitric oxide as an antipyretic in endotoxin-induced fever in rabbits was subsequently substantiated (Riedel, 1997). Studies conducted on gene knock-out mice have revealed differential roles of NOS isoforms in the fever of different etiologies (Kozak and Kozak, 2003). Like that of prostaglandins, species differences in the role of nitric oxide cannot be ruled out. For instance, nitric oxide has

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been found to have no modulatory effect on fever in cats (Redford et al., 1995).

Cyclooxygenase-2, the inducible isoform of the enzyme prostaglandin H synthase, has been reported to mediate the febrile response of mice to interleukin-1-beta (Li et al., 2001). Mice expressing the transgenic COX-2 in anterior hypothalamus revealed accelerated onset of fever in response to LPS compared to their non-transgenic counterparts (Vidensky et al., 2003). LPS-induced fever in rats has been reported to be associated with induction of COX-2 mRNA in the blood vessels and leptomeninges (Cao et al., 1997). Although the role of NOS and COX pathways in pyrogenic fever has been studied in rabbit and rat (Lin and Lin, 1996; Vayssettes-Courchay et al., 2002), yet it is not completely understood whether iNOS and COX-2 produce fever independent of each other or the two pathways partly contribute to some common mechanism in this aspect. Therefore, a study was planned to explore the nature of interaction between iNOS and COX-2 using *S*-methylisothiourea (selective iNOS inhibitor), rofecoxib (selective COX-2 inhibitor) and mefenamic acid (non-selective COX inhibitor) as experimental tools in Brewer's yeast-induced pyrexia in mice.

2. Materials and methods

2.1. Animals

This experiment was completed in five phases and accordingly five batches of adult male albino mice weighing between 25 and 30 g were procured from the Laboratory Animal Resource Section of the Institute. These mice were housed at controlled temperature (22±2 °C) and allowed free access to food and drinking water. The animals were acclimatized for a day before the actual experiment was conducted.

2.2. Drugs

Rofecoxib gifted by "Ranbaxy Laboratories India," *S*-methylisothiourea as its hemisulfate salt and mefenamic acid procured from "Sigma Chemical USA" were used in this study. *S*-methylisothiourea dissolved in distilled water, mefenamic acid dissolved in equimolar sodium hydroxide solution and rofecoxib in the form of aqueous suspension in 1% Tween-80 were orally administered to the experimental animals for plotting the dose–response curves.

2.3. Induction of pyrexia

After recording the rectal temperature of each of the animal in a batch, pyrexia was induced by subcutaneous injection of 20% Brewer's yeast suspension in the neck scruff @10 ml /kg body weight in the afternoon around 4.00 pm (Kupeli et al., 2002). Animals were fasted and 18 h later, on the following day, rectal temperature of each animal was recorded by use of a digital thermometer. The mice exhibiting a rise in temperature by more than 0.8 °C were considered as pyretic.

2.4. Recording of dose–response curve

The study involved recording of five dose–response curves, each for *S*-methylisothiourea, rofecoxib, mefenamic acid and combinations of *S*-methylisothiourea plus rofecoxib and *S*-methylisothiourea plus mefenamic acid. Recording of dose–response curve for each drug involved use of 36 animals divided randomly into six groups of six each. Five groups of animals were subjected to induction of pyrexia by Brewer's yeast, whereas one group served as naive control. Out of five pyretic groups, four groups were administered 4 different dose levels of the drug in question, whereas one group served as vehicle-treated pyretic control. Thus, for three drugs 36×3=108 animals were used in three phases. In a similar way, for recording the dose–response curve for each drug combination, out of five groups of six mice each, one group served as naive control, one served as vehicle-treated pyretic control and the remaining three groups were administered three different dose levels of the drug combination in question. Recording of dose–response curves for drug combinations involved use of 60 mice in total, using 30 mice for each drug combination. Thus, whole experiment was completed on 168 mice in five batches and each batch of animals had its own contemporary vehicle-treated pyretic and naive controls. Rectal temperature was recorded at 0, 1, 3, 5 and 7 h after the drug administration. For calculating the net response to a given dose of the drug in question over a period of 7 h, we computed an average for 1-, 3-, 5- and 7-h temperature records for each animal. The antipyretic efficacy (%) for a given dose of drug ,alone or in combination was calculated by using the formula:

$$\%\ \text{Efficacy} = [(\text{Average temperature of vehicle treated pyretic control}) - (\text{Average temperature of drug treated group})] / [(\text{Average temperature of vehicle treated pyretic control}) - (\text{Average temperature of naive control})] \times 100$$

2.5. Isobolographic analysis

The interaction of *S*-methylisothiourea with the given COX inhibitors was evaluated by simultaneous administration of fixed proportions of *S*-methylisothiourea with each COX inhibitor, and performing an isobolographic analysis as described by Tallarida et al. (1997). The isobologram was constructed by connecting the ED_{50} of the corresponding COX inhibitor, plotted on the abscissa with the ED_{50} of *S*-methylisothiourea plotted on the ordinate to obtain the additivity line. For each drug combination, ED_{50}, ED_{25} and $ED_{12.5}$ and the associated 95% confidence intervals were determined by linear regression analysis of the log–dose–response curve (6 animals at each dose) and

compared by '*t*'-test to the theoretical additive ED_{50}, ED_{25} and $ED_{12.5}$. For interaction studies, fixed-ratio proportions were selected by first combining the ED_{50} of each compound and then constructing a dose–response curve in which ED_{50} fractions (1/2, 1/4 and 1/8) of iNOS–COX inhibitor combinations were administered. Theoretical additive ED_{50}, ED_{25} and $ED_{12.5}$ of the combinations were determined by combining together 1/2 ED_{50} (e.g., 1/2 ED_{50} *S*-methylisothiourea+1/2 ED_{50} COX inhibitor), 1/2 ED_{25}, 1/2 $ED_{12.5}$ and 1/2 $ED_{6.25}$ of each drug and then following the linear regression analysis.

2.6. Statistics

Variances of additive combinations were calculated as per the procedure described by Pinardi et al. (2001).

$$\mathrm{VarED}_{x(\mathrm{add})} = (0.5)^2\ \mathrm{VarED}_{x(\text{iNOS inhibitor})} + (0.5)^2\ \mathrm{VarED}_{x(\text{COX inhibitor})}$$

where x is 50, 25, 12.5, or 6.25.

From the individual variances, 95% confidence limits were calculated for theoretical additive combinations. When the drug combination gives an experimental ED_{50} not statistically different from theoretically calculated ED_{50}, the interaction is said to be additive. Additivity means each constituent contributes to the effect according to its own potency and the less potent drug is acting as if it is merely a diluted fraction of the other (Tallarida, 2001).

Slopes of individual log–dose–response curves were compared by Student's '*t*-test' and theoretical additive line was compared to experimental combination lines by analysis of covariance as described by Tallarida et al. (1997). Theoretical effective doses were compared with experimental effective doses by Student's '*t*'-test.

3. Results

3.1. Antipyretic activity of S-methylisothiourea, rofecoxib and mefenamic acid

Average temperatures in degrees Celsius for various treatment groups along with that of contemporary vehicle-treated pyretic and naive control groups are summarized in Tables 1 and 2. Temperature before yeast injection ranged from 37.1 to 37.5 °C with an overall mean±SE of 37.2±0.02 °C (n=168). Temperature recorded 18 h after yeast injection ranged from 38.0 to 38.4 °C with an overall mean±SE of 38.2±0.01 °C (n=138). Temperature recorded for naive control mice 18 h after their contemporary controls were given yeast suspension injections ranged from 36.9 to 37.2 °C with an overall mean±SE of 37.1±0.2 °C (n=30). Diurnal variation of 0.23±0.02 °C (n=30) was recorded for the naive control mice with a range of 0.1–0.3 °C. No significant diurnal variation was found in vehicle-treated pyretic control. Per os administration of *S*-methylisothiourea, rofecoxib and mefenamic acid produced dose-dependent antipyretic effect in Brewer's yeast-induced pyrexia in mice, with different potencies. Peak antipyretic effect in *S*-methylisothiourea and rofecoxib treated animals was observed after 3 h of drug administration, whereas mefenamic acid administered animals exhibited the peak antipyretic effect after 1 h of the drug administration. Since the duration of antipyretic effect was about 7 h with each treatment, it was appropriate to calculate mean of 1-, 3-, 5- and 7-h temperatures for assessing the per cent efficacy of the drug in question. The dose–response data obtained for each drug alone and in combination, respectively are presented in Tables 1 and 2. Slopes of the dose–response curves of *S*-methylisothiourea, rofecoxib and mefenamic acid were 76.40±5.75; 46.40±9.80 and 53.50±9.60, respectively and were not statistically different from one another. ED_{50} values (mg/kg body weight) of *S*-methylisothiourea, rofecoxib and mefenamic acid were calculated to be 41.70±2.90; 1.79±0.61 and 8.20±2.10, respectively.

Table 1
Dose effect data for antipyretic efficacy of *S*-methylisothiourea, rofecoxib and mefenamic acid in adult male albino mice*

Experimental phase/animal batch	Treatment group	Average of 1-, 3-, 5- and 7-h temperatures±SE (°C)	Per cent efficacy
I	Naive control	37.20±0.03[b]	
	Vehicle-treated pyretic control	38.20±0.02[a]	
	S-methylisothiourea		
	25 mg/kg	37.90±0.03[c]	30
	50 mg/kg	37.60±0.03[d]	60
	100 mg/kg	37.40±0.03[e]	80
	200 mg/kg	37.20±0.01[d]	100
II	Naive control	37.20±0.04[b]	
	Vehicle-treated pyretic control	38.20±0.06[a]	
	Rofecoxib		
	1 mg/kg	37.90±0.08[d]	30
	3 mg/kg	37.50±0.06[c]	70
	10 mg/kg	37.30±0.07[b]	90
	30 mg/kg	37.20±0.08[b]	100
III	Naive control	37.10±0.03[de]	
	Vehicle-treated pyretic control	38.10±0.06[a]	
	Mefenamic acid		
	3 mg/kg	37.90±0.06[b]	20
	10 mg/kg	37.50±0.07[c]	60
	30 mg/kg	37.20±0.05[d]	90
	100 mg/kg	37.00±0.06[e]	100

Within a batch, values bearing different superscripts differ significantly (P<0.05); ANOVA followed by Studentized range test.
* n=6.

3.2. Interaction of S-methylisothiourea with rofecoxib and mefenamic acid

Points of theoretical additive line and experimentally derived line for interaction study of *S*-methylisothiourea

Table 2
Dose effect data for the combination of *S*-methylisothiourea with cyclooxygenase inhibitors*

Experimental phase/animal batch	Treatment group	Average of 1-, 3-, 5- and 7-h temperatures±SE	Per cent efficacy	
IV	*S*-methylisothiourea+rofecoxib			
	1/2 ED_{50} of each	21.70 mg/kg	37.60 ± 0.04^{c}	63.60
	1/4 ED_{50} of each	10.90 mg/kg	38.00 ± 0.07^{b}	27.30
	1/8 ED_{50} of each	5.40 mg/kg	38.20 ± 0.07^{a}	9.10
		Naive control	37.20 ± 0.05^{d}	–
		Vehicle-treated pyretic control	38.30 ± 0.09^{a}	–
V	*S*-methylisothiourea+mefenamic acid			
	1/2 ED_{50} of each	24.90 mg/kg	37.70 ± 0.10^{c}	58.30
	1/4 ED_{50} of each	12.50 mg/kg	38.10 ± 0.10^{b}	25.00
	1/8 ED_{50} of each	6.20 mg/kg	38.30 ± 0.05^{a}	8.30
		Naive control	37.20 ± 0.04^{d}	–
		Vehicle-treated pyretic control	38.40 ± 0.07^{a}	–

Within a batch, values bearing different superscripts differ significantly ($P<0.05$); ANOVA followed by Studentized range test.
* $n=6$.

with rofecoxib and *S*-methylisothiourea with mefenamic acid are summarized in Table 3. For either of the drug combination comparison of theoretical and experimentally derived lines using analysis of covariance technique indicated similarity in both slopes and intercepts.

Antipyretic activity induced by co-administration of fixed ratios of ED_{50} fractions of COX inhibitors and *S*-methylisothiourea was examined by the analysis of corresponding isobolograms. The effective doses for the response levels of 50%, 25% and 12.5% and their 95% confidence intervals are shown in Table 4. Isobolographic analysis for the combinations of *S*-methylisothiourea+rofecoxib and *S*-methylisothiourea+mefenamic acid revealed additive interaction as represented in Fig. 1A and B. In isobolograms, interaction is additive because 95% confidence interval of experimental ED_{50} of the combination overlapped the 95% confidence interval of theoretical ED_{50} of the corresponding drug combination.

4. Discussion

In Brewer's yeast-induced pyrexia in rodents, yeast cell wall products deposited subcutaneously act as exogenous pyrogens and stimulate the immune cells (macrophages and lymphocytes) to produce endogenous pyrogens in the form of cytokines in the circulation that go to anterior hypothalamus to alter the set point for body temperature (Miller, 2000). In the present study, all animals demonstrated pyrexia in response to Brewer's yeast. A dose-dependent antipyretic effect produced by *S*-methylisothiourea indicates that induction of inducible isoform of NOS in response to endogenous pyrogens is involved in the generation of pyrexia by the yeast cells. Such observations have also been reported in guinea pigs wherein *S*-methylisothiourea and aminoguanidine produced a dose-dependent attenuation of lipopolysaccharide-induced fever (Roth et al., 1999). Rofecoxib and mefenamic acid also produced dose-dependent antipyretic effects in mice in this model. This substantiates the role of cyclooxygenase-2 in yeast-induced fever in mice, because rofecoxib specifically inhibits COX-2 and mefenamic acid non-specifically inhibits COX. It is already reported that COX-2 isoform mediates febrile response of mice to interleukin-1-beta and COX-2 has predominant role in lipopolysaccharide-induced fever in rats (Li et al., 2001; Zhang et al., 2003). Therefore, it is inferred from the dose–response curves that iNOS and COX-2 are involved in pyrexia in response to Brewer's yeast.

Such inference is not surprising, since there are evidences that inducing insults for iNOS and COX-2 being similar, result in co-induction of these two enzymes

Table 3
Theoretical additive line and experimental combination line for combinations of *S*-methylisothiourea+rofecoxib and *S*-methylisothiourea+mefenamic acid

S-methylisothiourea+rofecoxib				*S*-methylisothiourea+mefenamic acid			
Theoretical additive line		Exptl. comb. line		Theoretical additive line		Exptl. comb. line	
Dose (mg/kg)	Per cent efficacy	Dose (mg/kg)	Per cent efficacy	Dose (mg/kg)	Per cent efficacy	Dose (mg/kg)	Per cent efficacy
21.70	50.00	21.70	63.60	24.95	50.00	24.95	58.30
10.10	25.00	10.90	27.30	11.20	25.00	12.48	25.00
6.90	12.50	5.40	9.10	7.55	12.50	6.24	8.30
5.70	6.25			6.20	6.25		
$F_{(calc)}=3.87$		$F_{(tab)}=10.13$ (P>0.05)		$F_{(cal)}=1.90$		$F_{(tab)}=10.13$ (P>0.05)	

Table 4
Theoretical effective doses, experimental effective doses and their 95% confidence intervals for combinations of *S*-methylisothiourea+rofecoxib and *S*-methylisothiourea+mefenamic acid

	S-methylisothiourea+rofecoxib		*S*-methylisothiourea+mefenamic acid	
	Theoretical	Experimental	Theoretical	Experimental
ED_{50}	21.70 (17.60–25.80)	16.50 (6.60–26.40)	24.95 (20.00–30.00)	21.40 (8.00–35.00)
ED_{25}	10.10 (7.00–13.20)	8.80 (4.50–13.10)	11.20 (7.80–14.60)	10.70 (5.60–15.80)
$ED_{12.5}$	6.90 (4.40–9.40)	6.40 (2.20–10.60)	7.55 (4.80–10.30)	7.60 (3.00–12.30)

(Bishop-Bailey et al., 1997; Hamilton and Warner, 1998). The most remarkable point, however, is the parallelism of the three dose–response curves as the slopes were not significantly different from one another. Applying the principles of biological assay, one can safely infer that some common mechanism is involved in the control of fever by *S*-methylisothiourea and rofecoxib or mefenamic acid in spite of the fact that these drugs inhibit different enzymes and hence, the production of different mediators. In other words, there occurs some cross-talk between iNOS and COX-2, finally leading to the generation of fever by some common pathway. It is already known that attenuation of fever by iNOS inhibitors is independent of the circulating cytokine network (Roth et al., 1998b). Further, fever originates by central nervous system activities, but neither exogenous nor endogenous pyrogens are able to cross the blood brain barrier. It is, therefore, certain that some freely diffusible molecular species must be acting as mediator for genesis of fever in response to yeast. From the last few years, search is being made to unravel the putative role of reactive oxygen and reactive nitrogen oxide species as signalling molecules in the genesis of fever. It has recently been propounded that oxidative stress is being sensed by redox-sensitive site of the *N*-methyl-D-aspartate receptor for glutamate followed by oxidation of the site and development of fever (Nomoto et al., 2004). This is based on the observation that oxygen radical scavengers and thiol reductants act as antipyretics. Inhibition of oxygen radical formation by methylene blue, aspirin, or α-lipoic acid has been reported to prevent bacterial lipopolysaccharide-induced fever not only in rabbits (Riedel et al., 2003) but also in pigeons (Nomoto and Riedel, 2004).

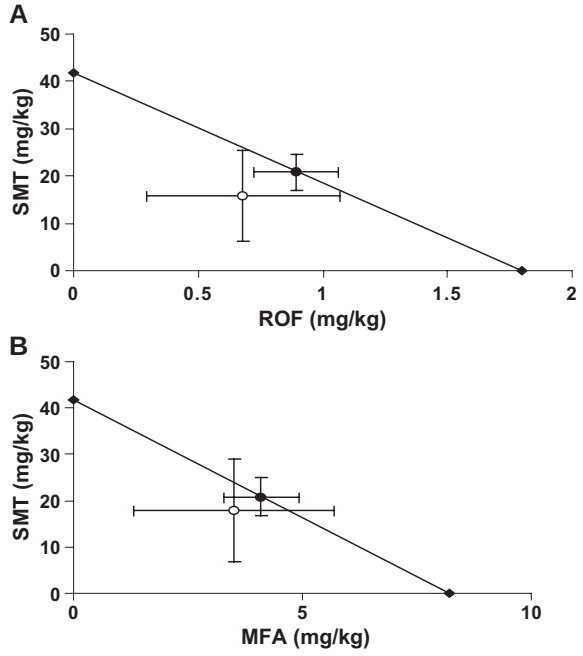

Fig. 1. Isobologram for the simultaneous administration of *S*-methylisothiourea and rofecoxib, (A) *S*-methylisothiourea and mefenamic acid, (B) filled circles represent theoretical ED_{50} with 95% confidence limits and open circles represent the experimental ED_{50} with 95% confidence limits. Ordinates and abscissae are on different scales.

From the findings of our experiment, it seems true that iNOS and COX-2 complement each other in such a manner, that inhibition of either of the two systems prevents development of fever. This is because *S*-methylisothiourea, rofecoxib and mefenamic acid when given alone could effectively reduce fever and that too with 100% efficacy. It has been shown that iNOS inhibition blocks prostanoid production by 50% in rats, thereby signifying the regulatory influence of nitric oxide on COX (Salvemini et al., 1995). Further, iNOS–COX pathways were also explored for their possible role in pyrogenic fever in rabbits (Lin and Lin, 1996), but the design of this study has been such that the individual roles of iNOS and COX-2 have been well ascertained without addressing the nature of their interaction. However, the present study is based on isobolograms and comparison of composite additive curve with experimental combination curve is considered as the efficient design for assessing the nature of interaction (Tallarida et al., 1997; Tallarida, 2001). Isobolographic analysis revealed no significant departure of experimentally derived ED_{50} from the theoretically additive ED_{50} for both *S*-methylisothiourea+rofecoxib and *S*-methylisothiourea+-mefenamic acid combinations. This is possible only if nitric oxide and prostanoids follow an additive type of interaction in yeast-induced pyrexia in mice. But, this cannot be achieved unless the production of signaling mediator molecules is subservient to both iNOS and COX-2. Therefore, from this study, it is concluded that interaction between iNOS and COX-2 is complementary and additive in nature. However, further investigations are required to ascertain whether peroxynitrite ion ($ONOO^-$), that is usually formed by the rapid reaction of nitric oxide with even low concentrations of superoxide anion (O_2^-), does or

does not function as a signalling molecule in the generation of fever.

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